Photoinactivation of Photosystem II and Degradation of the D1 Protein are Reduced in a Cytochrome b$_{6}$/f-Less Mutant of Chlamydomonas reinhardtii

Susana Shochat$^a$, Noam Adir$^a$, Alma Gal$^a$, Yorinao Inoue$^b$, Laurence Mets$^c$, and Itzhak Ohad$^a$

$^a$ Department of Biological Chemistry The Hebrew University of Jerusalem, Jerusalem, Israel
$^b$ The Solar Energy Group, Institute of Physical and Chemical Research (RIKEN), Wako-Shi, Saitama, Japan
$^c$ Department of Biology, University of Chicago, Chicago, U.S.A.

Z. Naturforsch. 45c, 395–401 (1990); received November 24, 1989

Cytochrome b$_{6}$/f, Chlamydomonas, D1 Turnover, Q$_{b}$, Thermoluminescence, Photoinhibition

The effect of unoccupancy of the Q$_{b}$ site by plastoquinone on the photoinactivation of reaction center II in a Cyt b$_{6}$/f-less mutant of Chlamydomonas reinhardtii, B$_{c}$, was investigated. In these cells the oxidation of plastoquinol generated by electron flow via RC II to plastoquinone and thus the turnover of PQH$_{2}$/PQ via the Q$_{b}$ site are drastically reduced. Reaction center II of the mutant cells was resistant to photoinactivation relative to the control cells as demonstrated by measurements of light-induced destabilization of S$_{a}$-Q$_{b}$ charge recombination, rise in intrinsic fluorescence and loss of variable fluorescence. These parameters relate to functions involving the reaction center II D1 protein. The light-induced degradation of D1 in the mutant cells was also considerably reduced, with a 1/2 value of 7 h as compared, under similar conditions, to about 1.5 h for the control cells. These results indicate that the photoinactivation of RC II and turnover of the D1 protein are related and require occupancy of the Q$_{b}$ site by PQ and its light-driven reduction.

Introduction

It is now well established that the D1 protein, one of the major components of the photosynthetic reaction center II [1], is rapidly degraded and resynthesized (turnover), when oxygen evolving photosynthetic membranes of both eukariotic and prokariotic organisms are exposed to light in vivo (reviewed in [2] and [3]). Light fluency exceeding that required to saturate photosynthesis, causes a gradual inactivation of photosystem II (reviewed in [2] and [4]), (photoinhibition). Recovery from extensive photoinhibition, requires de novo synthesis of chloroplast translated proteins, specifically that of the reaction center II protein D1 [5]. We have proposed previously that the process of photosystem II photoinactivation and turnover of the D1 protein are related phenomena [6, 7]. Recently we have obtained experimental evidence for the involvement of the Q$_{b}$ binding site located within the D1 protein [8, 9], in this process [6, 7]. Using the thermoluminescence technique for characterization of the charge recombination of the S states and Q$_{b}$ [10, 11], it was demonstrated that exposure of intact Chlamydomonas cells to light intensities saturating photosynthesis induces a destabilization of the reduced semiquinone Q$_{b}$ compatible with a change in its redox potential and shortening of its life time, [6]. These changes correlated with a reduction in the oxygen evolution capacity and coincided with the loss of variable fluorescence characteristic of photoinhibition and with an increase in the turnover of the D1 protein which persisted even when the cells were transferred to lower light intensities [6, 7].

Participation of the Q$_{b}$ semiquinone in these processes requires occupancy of the Q$_{b}$ site of RC II by plastoquinone. Arguments in favor of this hypothesis could be found in the observation that herbicides binding at the same site such as Diuron prevents the D1 protein light-dependent degradation in vivo [12] and the light-induced destabilization of Q$_{b}$ [7]. As opposed to that, in vitro experiments in which thylakoids were depleted of plastoquinone showed an increased sensitivity of the D1 protein to proteolysis by trypsin. These results were considered as an indication for an enhanced susceptibility of the D1 toward degradation when the Q$_{b}$ site is unoccupied by plastoquinone [13]. In the present work we have used a

Abbreviations: Chl, chlorophyll; Cyt b$_{6}$/f, Cytochrome b$_{6}$/f complex; $F_{0}$, $F_{s}$, $F_{m}$, intrinsic, steady-state and maximal fluorescence, respectively; PQ, plastoquinone; POQH$_{2}$, plastoquinol; PS II, photosystem II; RC II, reaction center II; TL, thermoluminescence.

Reprint requests to I. Ohad.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen 0341–0382 90/0500–0395 $01.30/0

Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung 4.0 Lizenz.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution 4.0 International License.
cytochrome \(b_6/f\)-less mutant of \(Chlamydomonas\) to measure the light-dependent turnover of the D1 protein and stability of the RC II toward photoinactivation. In such a mutant the plastoquinone pool should be mostly reduced in the light to plastoquinol, a condition which favors unoccupancy of the Q\(_b\) binding site. The results clearly demonstrate that in this mutant RC II is protected against the light-induced D1 turnover and photo-inhibition.

**Materials and Methods**

\(Chlamydomonas\) reinhardtii \(y-1\) and the \(B_6\) mutant cells were grown in a liquid medium containing acetate as a carbon source as described before [14]. The \(y-1\) mutant does not synthesize chlorophyll in the dark but is indistinguishable from the wild type cells when grown in the light. Light-grown cells are photosynthetically competent and exhibit normal photosynthetic electron flow [14]. \(B_6\) is a chloroplast mutant lacking Cyt \(b_6/f\) activity [15]. As reported before for other cytochrome \(b_6/f\)-less mutants [16–18], this mutant does not exhibit a light-dependent or redox-dependent light harvesting II complex protein phosphorylation [19].

For measurements of D1 protein degradation, cells were incubated at the light intensities and times as indicated in the presence of chloramphenicol (200 \(\mu\)g/ml) added to prevent synthesis of the chloroplast translated D1. Thylakoid membranes were prepared as described before [20], and protein immunodetection using various antibodies was performed after resolving the thylakoid proteins by SDS-PAGE as described by Laemmli [21] followed by transfer to nitrocellulose paper and detection of the immunodecorated polypeptides by alkaline phosphatase or \(\gamma\) iodinated protein A.

Thermoluminescence measurements were performed on intact cells or isolated thylakoids obtained from cells exposed to various light treatments as previously described [6].

Fluorescence rise kinetics was recorded using a home-made apparatus consisting of a cell holder, excited by a DC light source equipped with an electronic shutter (opening time, 2 msec) and filtered through a blue Corning 4-96 filter. The signal was detected at a right angle by a photodiode protected by a 685 nm cut-off red filter (Schott). The signal was digitized, stored and analyzed by an IBM-PC computer programmed to operate the shutter and record the voltage rise at different rates (up to 30000 points/sec) so as to cover efficiently both the fast and slow phase of the fluorescence rise.

Photoinhibitory light was provided by a projector equipped with a heat and UV filter (Pro Cabin, 680 W Tungsten-halogen lamp) supplying about 80% of the light energy in the visible region. The incubation vessel was temperature controlled by a surrounding water jacket (25 °C) as described before [6] and Chl concentration was 30–35 \(\mu\)g/ml.

**Results**

**Effect of high light treatment on the fluorescence kinetics of the \(B_6\) mutant cells**

The absence of the cytochrome \(b_6/f\) polypeptides in the mutant as demonstrated by immunoblotting with antibodies against cytochrome \(b_6\), cytochrome \(f\) and the Rieske polypeptide is shown in Fig. 1. Comparison of the thylakoid polypeptide pattern of the mutant to that of the \(y-1\) cells did not disclose other significant differences. To assay the sensitivity of the \(b_6\) mutant relative to that of the \(y-1\) cells to high light intensities, cell suspensions were incubated in growth light or exposed to 250 \(\text{Wm}^{-2}\) for 30 min and the fluorescence rise kinetics of both types of cells was recorded after preadapting them to the dark for 1 min [6, 7]. The \(y-1\) cells in which electron flow is uninhibited, showed a low steady state fluorescence \((F_s)\) as compared to the maximal fluorescence exhibited in the presence of the photosystem II inhibitor, diuron \((F_{max}, \text{Fig. 2})\). As expected, the steady-state
A high rate of oxygen-dependent oxidation of plastoquinol could permit continuous reduction of plastoquinone in the light-exposed cells and thus increase at least to some extent the occupancy of the Qb site by plastoquinone even in absence of an active cytochrome b6/f complex. To estimate the rate of plastoquinol reoxidation the effect of the dark adaptation time following light exposure of cells on the fluorescence rise kinetics in the Bb mutant cells was assayed. The results of such an experiment are shown in Fig. 3. Mutant cells were incubated in low or high light and then the fluorescence rise kinetics was measured repeatedly after short dark adaptation time intervals. In low light exposed cells, the fluorescence rise kinetics after 15 sec of dark adaptation was comparable but not identical to that obtained following dark adaptation for 1 min indicating that part of the plastoquinone pool was still reduced. In cells exposed to high light the plastoquinone pool remained partially reduced even after 1 min of dark adaptation of the preilluminated cells (Fig. 3). These results

fluorescence level, Fv, of the Bb mutant was similar in the absence or presence of diuron, since the mutant can not oxidize plastoquinol. However the rise time of the Fv level in the Bb mutant is considerably slower than that of the Fmax level due to the time required for the reduction of the plastoquinone pool in the light (Fig. 2). The variable fluorescence, (Fv), calculated as (Fmax−F0)/Fv, indicative of the relative content of active RCIII, was higher in the y-l cells as compared to the Bb mutant due to a higher level of intrinsic fluorescence (F0) in the mutant cells (Fig. 2). Following exposure to high light, the variable fluorescence in the y-l cells decreased significantly, due to a rise in the F0 level and reduction of the Fmax, as previously reported for photoinhibited cells [4, 6, 7]. No such reduction in the Fv level was observed however in the Bb mutant cells, indicating that these cells were not photoinhibited by this treatment (Fig. 2).

It was previously reported that in Chlamydomonas cells chlororespiration activity can account for the oxidation of the plastoquinol pool [22, 23].
indicate that the B₆ mutant can oxidize plastoquinol in vivo, however the rate of this reaction is slow and further reduced in cells which have been exposed to high light intensity.

**Effect of high-light exposure on the thermoluminescence glow curves of the y-1 and B₆ mutant cells**

Charge recombination between the S₂,3 states and the primary or secondary stable reduced acceptors, Qₐ or Q₈, can be elicited by heating in the dark, isolated chloroplasts or whole cells, after preillumination by a saturating single turnover light flash in the presence or absence of diuron respectively [10, 11]. The recombination results in light emission and a glow curve (thermoluminescence), can be obtained in which the intensity of the light emitted as a function of the sample temperature is recorded. Under specific experimental conditions, the temperature at which the emission is maximal is characteristic of the recombining species, and related to the redox potential or their stability [10, 11]. Charge recombination between the S states and Q₈ (B band [10, 11]), in intact *Chlamydomonas* cells is characterized by a maximal light emission at about 37–39 °C while that resulting from the recombination of Qₐ (D band [10, 11]), peaks at about 7–10 °C (Fig. 4, [6, 7]). Similar results were obtained with isolated thylakoids (data not shown). It has been shown before that the B band emission peak is shifted to a lower temperature, comparable to that of the D band in cells [6, 7], or thylakoids [7] isolated from cells which have been exposed to photoinhibitory light. Recent experimental results demonstrate that this shift in the B band temperature peak is indicative of changes in the D₁ protein properties, and correlated with the loss of reaction center II activity and the light-dependent D₁ protein synthesis [6] and modification [7]. Thus thermoluminescence measurements could provide information on the light-induced changes in the reaction center II of the B₆ mutant as compared to the y-1 cells. Results of such experiments are shown in Fig. 5. The peak temperature of the B band emission is reduced in y-1 cells as a function of exposure to high-light intensity. The temperature shift correlates with loss of variable fluorescence due to rise in the F₀ and decrease in the Fₘₐₓ values (Table I, see also Fig. 5) as reported before [6, 7]. In contradistinction, the glow curve of the B₆ mutant cells shows a broad emission temperature including a shoulder at about 16 °C which is shifted to a higher temperature following high-light treatment. This indicate that prior to the high light exposure part of the reaction centers of the mutant cells are already altered and the glow curve shape is due to the presence of a mixed population of intact and altered reaction centers which revert to the unaltered form during the high-light exposure.

**Table I. Resistance in vivo to light-induced damage of PS II in the B₆ mutant.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells</th>
<th>F₀ (%) of control</th>
<th>Fₘₐₓ/F₀</th>
<th>Δ °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>125 Wm⁻²; 50 min</td>
<td>y-1</td>
<td>240</td>
<td>12</td>
<td>-21</td>
</tr>
<tr>
<td></td>
<td>B₆</td>
<td>65</td>
<td>157</td>
<td>-4</td>
</tr>
<tr>
<td>250 Wm⁻²; 30 min</td>
<td>y-1</td>
<td>190</td>
<td>24</td>
<td>-24</td>
</tr>
<tr>
<td></td>
<td>B₆</td>
<td>76</td>
<td>118</td>
<td>+10</td>
</tr>
</tbody>
</table>

Δ °C, shift in the emission temperature of S₂-Q₈ charge recombination; for experimental details, see Methods.
Since the \(B_6\) mutant cells exhibited an anomalous glow curve showing a lower emission temperature peak of the B band in low light exposed cells which shifted to a higher temperature in high light exposed cells, it was of interest to test whether this phenomenon is correlated with the total amount of absorbed energy. Thus the effect of light intensity and time of exposure on the kinetics of the changes in the thermoluminescence and fluorescence parameters was assayed. Results of such an experiment are shown in Fig. 5. A clear tendency is demonstrated toward an improved response to higher light intensities in terms of the stability of the thermoluminescence and fluorescence parameters in the \(b_{il-f}\)-less mutant cells as compared to the \(y-1\) control cells.

**Light-induced turnover of the D1 protein**

The results presented so far demonstrate that photosystem II of the cytochrome \(b_{il-f}\)-less mutant is resistant to the light-induced loss of reaction center II activity relative to that of the \(y-1\) cells as demonstrated by the reduced temperature peak shift of the B band thermoluminescence signal and the persistence of the variable fluorescence in the high light-treated cells. If indeed the light-induced turnover of the D1 protein is related to the above light-induced changes in reaction center II, one would predict a significant reduction in the light-dependent degradation of the D1 protein in the \(B_6\) mutant cells. To test this possibility, \(y-1\) and \(B_6\) mutant cells were exposed to high light intensity for various times in the presence of chloramphenicol to prevent the de novo synthesis of the D1 protein and thus allow the detection of the light-dependent loss of the D1 as assayed by protein immunoblotting. Results of such experiments are shown in Fig. 6 and demonstrate unequivocally the resistance of the D1 protein in the \(B_6\) mutant to the light-dependent degradation in vivo as com-

![Fig. 5. Effect of light exposure on the thermoluminescence emission temperature and fluorescence rise. Cell suspensions were incubated at low growth light intensity (25 Wm\(^{-2}\)) for 30–60 min and the time 0 sample were taken; the cells were then transferred to light intensities as indicated and samples were taken at various times for measurements of thermoluminescence and fluorescence parameters as described in the Methods; the glow curves of the \(B_6\) mutant cells were asymmetric (cf. Fig. 4) indicating the presence of a population of unaltered reaction centers displaying an emission temperature as in control samples (values in parenthesis); note the gradual reduction in the effect of time exposure on the TL temperature peak and loss of variable fluorescence in the \(B_6\) mutant cells exposed to higher light intensity as compared to the \(y-1\) control cells.](image1)

![Fig. 6. Light-dependent degradation of the D1 protein is significantly reduced in the \(B_6\) mutant cells. Cell suspension were exposed to 250 Wm\(^{-2}\). At times as indicated samples were taken and the residual amount of D1 protein in isolated thylakoids was detected by use of \(^{125}\)iodinated protein A. The estimated t 1/2 value of D1 was about 1.5 and 7 h in the \(y-1\) and the \(B_6\) mutant cells respectively.](image2)
pared to that of the γ-I cells. The relative amounts of the D1 protein on a chlorophyll basis indicates that the amount of this protein (and thus of RC II) is somewhat lower in the mutant as compared to the γ-I cells. This could imply that the ratio antenna/RC II is higher and thus more energy is absorbed by RC II in these cells.

**Discussion**

The mechanism whereby light causes the degradation of the reaction center II D1 protein is not yet understood. Furthermore the relation of this phenomenon to that of the light-dependent inactivation of photosystem II in vivo (photoinhibition), is a controversial issue [4, 24]. In our working hypothesis the two phenomena are considered as related [7]. It has been proposed before [2, 4, 5] that electron flow via the Q₂ secondary acceptor quinone of reaction center II may induce reversible changes in the conformation of the reaction center complex which could facilitate formation of harmful free radical species. These could be oxygen or hydroxyl radicals as well as the semiquinone anion [2, 4, 5, 25] or the primary donor, Z [7]. These could cause further specific irreversible covalent changes in the D1 protein resulting in its exposure to proteolytic degradation. Reversible light-induced conformational change in reaction center II affecting primarily the redox/stability of the Q₂ have been recently demonstrated to occur in vivo [7], and found to correlate with the initial loss of variable fluorescence and partial loss of reaction center II activity. Furthermore under these conditions we could also demonstrate that a fraction of the D1 protein undergoes a covalent modification [7], which correlates with an increase in the turnover of this protein [26]. The above hypothesis is supported by the findings that reversible conformational changes affecting the redox potential of the acceptor quinone are induced by light in isolated bacterial reaction centers in which the D-Q₂ species have been generated [27]. The presence of a protease sensitive “PEST” sequence [25], specific to the D1 protein which could be the primary site of degradation of this protein in vivo supports the concept that in the irreversibly modified protein this sequence became accessible to proteolysis. However the validity of our hypothesis rest on the demonstration that indeed occupancy of the Q₂ site by plastoquinone and thus the ensuing light-dependent electron flow via this site is required for these phenomena to occur. The data presented here strongly support the contention that indeed continuous PQ/PQH₂ turnover within the Q₂ site is essential for the light-induced loss of reaction center II activity and degradation of D1 protein. In the mutant lacking the cytochrome b₆/f complex, light-dependent electron flow via reaction center II is rate-limited by reoxidation of the plastoquinol due to chlororespiration [22, 23]. This process is apparently slow as indicated by the data presented here and may account for the observed partial sensitivity to the light of the B₆ mutant reaction center II. In cells exposed to higher light fluence the mechanism(s) responsible for the plastoquinone oxidation, for reasons not yet understood, became less efficient and thus the occupancy of the Q₂ site is further reduced. In these cells, the light-induced loss of reaction center II activity and the downshift in the TL emission temperature are less affected than in cells exposed to intermediate light intensity and may account for the observed “recovery” of TL signal temperature in B₆ cells transferred from low to high light (Fig. 3, 5).

It has been reported previously that in Spirodea ultra-violet (UV B) light causes enhanced degradation of the D1 protein as compared to that induced by similar fluency of photosynthetic light [28]. This may be explained if one considers that in cells exposed to UV light direct excitation of the bound quinone could induce the specific modification of the D1 protein only when the site is occupied. Degradation of the D1 protein was elicited also by far red light which supported photosystem I cyclic electron flow but only a limited photosystem II activity [29]. In this case, generation of plastoquinol will be rate limiting relative to its oxidation by photosystem I and occupancy of the Q₂ site by plastoquinone will be favored. The light-induced modification of reaction center II which appears to affect specifically the D1 protein as detected by the thermoluminescence measurements and loss of variable fluorescence, is also prevented partially in cells exposed to the light in the presence of DBMIB [7], an inhibitor which at low concentrations blocks electron flow via cytochrome b₆/f [30] and thus favors accumulation of plastoquinol as in the case of the B₆ mutant. Based on the data presented here and elsewhere [7, 26] we
thus conclude that the light-dependent degradation of the D1 protein is related to the loss of reaction center II activity. The mechanism of this phenomenon involves sustained turnover of the reduced/oxidized plastoquinone via the Q_B site.

Additional Chlamydomonas mutants lacking the cytochrome b_{6/}f complex, plastocyanin or photosystem I (manuscript in preparation) show a similar reduction in the light-dependent turnover of D1 protein. Experiments devised to further elucidate the role of plastoquinol oxidation rate in the turnover of D1 protein are now in progress.

Acknowledgements
This work was supported by grant 199 from the Israeli Academy of Science and SFB 184-87, from F.R.G. Antisera against cytochrome b_{6/}f subunits was kindly supplied by Dr. G. Hauska, University of Regensburg, F.R.G.